Neuron, Vol. 27, 121–131, July, 2000, Copyright ©2000 by Cell Press

Potentiation of Neuronal L Calcium Channels by IGF-1 Requires Phosphorylation of the α1 Subunit on a Specific Tyrosine Residue

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Summary

Insulin-like growth factor 1 (IGF-1) rapidly potentiates N and L calcium channel currents in cerebellar granule neurons by an unknown mechanism. Here, we show that the L channel α1C subunit is tyrosine phosphorylated in response to IGF-1. Moreover, expression of kinase-dead c-Src in neurons or acute block of Src family kinases with a cell-permeable inhibitor specifically blocks L channel potentiation. Purified Src kinase phosphorylates tyrosine residue Y2122 of the C terminus of neuronal α1C in vitro, and c- and v-Src directly bind the C terminus. When expressed in neuroblastoma cells, point mutation of Y2122 prevents both tyrosine phosphorylation of α1C and IGF-1 potentiation. Our data provide a biochemical mechanism whereby phosphorylation of a single specific tyrosine residue rapidly modifies ion channel physiology.

Introduction

Although previously considered as mitogens with long-term effects, growth factors, such as insulin-like growth factor 1 (IGF-1), have now also been shown to be rapid neuromodulators. IGF-1 regulates ion channel currents and neuronal excitability (Selinfreund and Blair, 1994; Blair and Marshall, 1997; Blair et al., 1999a) and is of particular interest in the central nervous system, where it is synthesized and widely distributed (Bondy et al., 1992). The IGF-1 receptor is present in both developing and mature neurons (Kar et al., 1993), suggesting an initial role in differentiation and a subsequent role in survival (D'Ercole et al., 1996; Stewart and Rotwein, 1996). Indeed, IGF-1 mediates the survival of granule neurons, partially through L calcium channel modulation (Blair et al., 1999a).

Understanding the signaling pathways regulating IGF-1 modulation of neuronal channels is essential to providing insight into the regulation of neuronal excitability and survival. We previously showed that IGF-1 rapidly potentiates neuronal L channels via a PI-3 kinase– and akt-dependent pathway (Blair and Marshall, 1997; Blair et al., 1999a). Other cellular kinases and phosphatases can also influence channel activity (reviewed by Levitan, 1994; Conley and Brammar, 1999). In the central nervous system, protein tyrosine kinases (PTKs) have been shown to be critical; of the Src family PTKs, Src, Fyn, Yes, and Lyn are the most abundantly expressed (Sudol et al., 1988; Wagner et al., 1991; Umemori et al., 1992). Significantly, AMPA receptors associate with Lyn (Hayashi et al., 1999), and NMDA receptors, as well as Na^+ and K^+ channels, can be regulated by Src (Holmes et al., 1996; Kohr and Seeburg, 1996; Yu et al., 1997; Fadool and Levitan, 1998; Hilborn et al., 1998).

Calcium channels are also regulated by Src. Src increases calcium currents in smooth muscle, the muscle L channel α1 subunit coprecipitates with Src, and Src inhibition can reduce L currents in several cell types (Cataldi et al., 1996; Wijetunge and Hughes, 1996; Strauss et al., 1997; Hu et al., 1998). Interestingly, regulation of neurotransmitter release in cerebellar neurons by L channels involves Src family–mediated tyrosine phosphorylation (Evans and Pocock, 1999). Our results show that IGF-1 potentiation of L channel activity ultimately requires phosphorylation of a specific tyrosine residue in the α1C C terminus, providing a novel mechanism of rapid growth factor modulation of neuronal L channels.

Results

The α1C Subunit Increases in Phosphotyrosine in Response to Acute IGF-1 Stimulation

The unique voltage sensitivity of L channel potentiation by IGF-1 in granule neurons, as well as the rapid action of IGF-1, suggests that existing channels are being biochemically modified (Blair and Marshall, 1997). Since several ion channels are known to be modulated by PTKs, we tested whether physiological levels of IGF-1 (50 ng/ml) could change the phosphotyrosine content of endogenous or heterologously expressed α1C subunits. In Figure 1A, endogenous α1C subunits were immunoprecipitated from solubilized granule cell membranes either before or after acute IGF-1 treatment with an antibody against the II–III linker region of the cardiac α1C subunit (CARDI; Chien, et al., 1995). This region corresponds to amino acids 785–902 of the rat brain isoform. As shown previously (Hell et al., 1993; De Jongh et al., 1996), we observe two size forms, a full-length protein with an apparent molecular mass of 240 kDa and a C-terminal truncated isoform of 210 kDa (Figure 1A, left panel, lanes 2 and 3). The α1C antibody immunoprecipitated both, while control preimmune serum did not (Figure 1A, left panel, lane 1). Immunoprecipitated channel subunits were probed by Western blot with a phosphotyrosine antibody. As shown in Figure 1A (right panel), only the full-length α1C subunit increases phosphotyrosine content in response to IGF-1.

Although granule neurons provide a suitable system for electrophysiological studies, limited material makes biochemical experiments impractical. Therefore, to reproduce IGF-1/L channel modulation in a neuronal cell line susceptible to manipulation, we constructed a c-Myc-tagged rat brain α1C subunit, placing the c-Myc tag at the C terminus of the full-length α1C to ensure that only the nontruncated isoform would be detected. α1C was coexpressed by transient transfection with the rat brain α2δ and β1b subunits in the human neuroblastoma cell line SH-SY5Y. These cells express IGF-1 receptors

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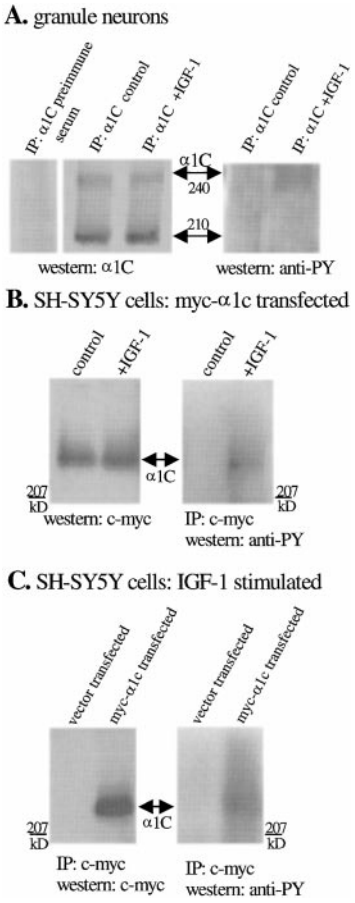

Figure 1. α1C Phosphotyrosine Content Increases in Response to IGF-1 in Both Granule Neurons and Transfected SH-SY5Y Neuroblastoma Cells

(A) Western blot analysis with an anti-phosphotyrosine antibody (right panel) shows that the endogenous full-length 240 kDa α1C subunit immunoprecipitated from granule neurons increases in phosphotyrosine in response to acute IGF-1 stimulation, while the C-terminal truncated 210 kDa isoform does not. Cerebellar granule neurons in culture were stimulated with IGF-1 (50 ng/ml) or vehicle ("control") for 3 min and α1C subunits immunoprecipitated from solubilized membranes using an antibody against amino acids 785–902 of endogenous L-type channels (CARDI). Left panel indicates control Western blot with CARDI showing that preimmune serum did not nonspecifically precipitate either form of the α1C subunit (lane 1) and that equal amounts of α1C were present in each test condition (lanes 2 and 3); n = 3 independent experiments.

(B) Similarly, acute IGF-1 stimulation increases the phosphotyrosine content in heterologously expressed neuronal α1C subunits. SH-SY5Y human neuroblastoma cells were cotransfected with c-Myc-tagged α1C, α2δ, and β1b subunits and stimulated with IGF-1 as in (A), and the α1C subunit was immunoprecipitated from solubilized membranes using an anti-c-Myc antibody. To ensure that only the nontruncated isoform would be detected, the c-Myc tag was placed at the C-terminal of the full-length α1C. Again, the left panel shows a control Western blot to demonstrate that equal amounts of α1C from each condition were used. The right panel shows the IGF-1-induced increase in phosphotyrosine levels; n = 5 independent experiments.

(C) To demonstrate the specificity of the c-Myc antibody for the tagged α1C, we performed immunoprecipitations from both vector-transfected and c-Myc-tagged α1C cells. All cells were stimulated with IGF-1 as in (A). Left panel, the anti-c-Myc antibody only precipitates a full-length α1C from the transfected cells. Right panel, increases in phosphotyrosine content are detected only in α1C-transfected cells.

and neuronal markers (Matthews and Feldman, 1996; Singleton et al., 1996) and have negligible L channel currents (see below; Reeve et al., 1994: Hirota and Lambert, 1997). We find that SH-SY5Y cells endogenously express PI 3 kinase and akt, the known elements of the IGF-1/L channel signaling pathway (Blair and Marshall, 1997; Blair et al., 1999a), as well as c-Src, and that the c-Myc-tagged α1C forms functional channels with normal L current characteristics (see Figure 7). We also find that heterologously expressed α1C increases in phosphotyrosine in response to acute IGF-1 stimulation (Figure 1B, right panel). The ability to immunoprecipitate specifically the c-Myc-tagged α1C was confirmed using vector-transfected cells (Figure 1C).

IGF-1 Rapidly Modulates Granule Neurons' L Channels via a Src Family Kinase

Our previous studies demonstrated that IGF-1 strongly potentiates neuronal L and N channel activities at specific membrane voltages, increasing N currents at depolarized potentials and L currents at hyperpolarized potentials (Blair and Marshall, 1997). Given that the muscle L channel is acutely regulated by Src (Wijetunge and Hughes, 1996) and our finding that neuronal α1C is tyrosine phosphorylated, the potential role of a Src tyrosine kinase in rapid, IGF-1-modulation was evaluated.

We first employed a "dominant-negative"approach (Blair and Marshall, 1997), analyzing neurons transiently transfected with c-Src (normal cellular), v-Src (constitutively active), or kinase-dead Src (a K295M mutation in the ATP binding domain of c-Src renders it catalytically inactive; Kmiecik and Shalloway, 1987). To identify transfectants, Src isoforms were coexpressed with the jellyfish green fluorescent protein (GFP; Marshall et al., 1995). Results were then compared with those obtained using acute application of active and inactive analogs of Src family kinase inhibitors.

Overexpression of kinase-dead Src, but not wild-type c-Src, prevented the rapid IGF-1 potentiation of L channel currents. Specifically, potentiation occurred in all neurons overexpressing c-Src (Figures 2A, 2E, and 2F; n = 18 of 18 cells), with IGF-1 inducing large increases at hyperpolarized membrane potentials that exceeded those of untransfected neurons (Figures 2B and 2F; n = 9 of 9 cells) but having, as expected, little or no effect at depolarized potentials (Figures 2E and 2F; Blair and Marshall, 1997). However, when neurons overexpressing kinase-dead Src were tested, the potentiation typically observed at hyperpolarized potentials was fully blocked (Figures 2C and 2F; n = 34 of 34 cells). As an internal control for cellular viability and possible nonspecific effects of Src overexpression, N channel potentiation, which occurs through a parallel pathway (Blair et al., 1999a), was examined to ensure that the IGF-1 signaling pathways were otherwise intact. As seen in Figures 2A and 2C, overexpression of Src isoforms did not affect IGF-1-induced N channel potentiation (c-Src, n = 5 of 5 cells; kinase-dead Src, n = 16 of 16 cells), which occurs primarily at depolarized potentials and is absent at strongly hyperpolarized potentials (Blair and Marshall, 1997).

Identical results were obtained when the Src family kinase inhibitor PP2 (Hanke et al., 1996) was used to block endogenous c-Src activity. Because PP2 is highly membrane permeable, Src family kinases can be acutely inhibited, thus averting potential nonspecific conse-

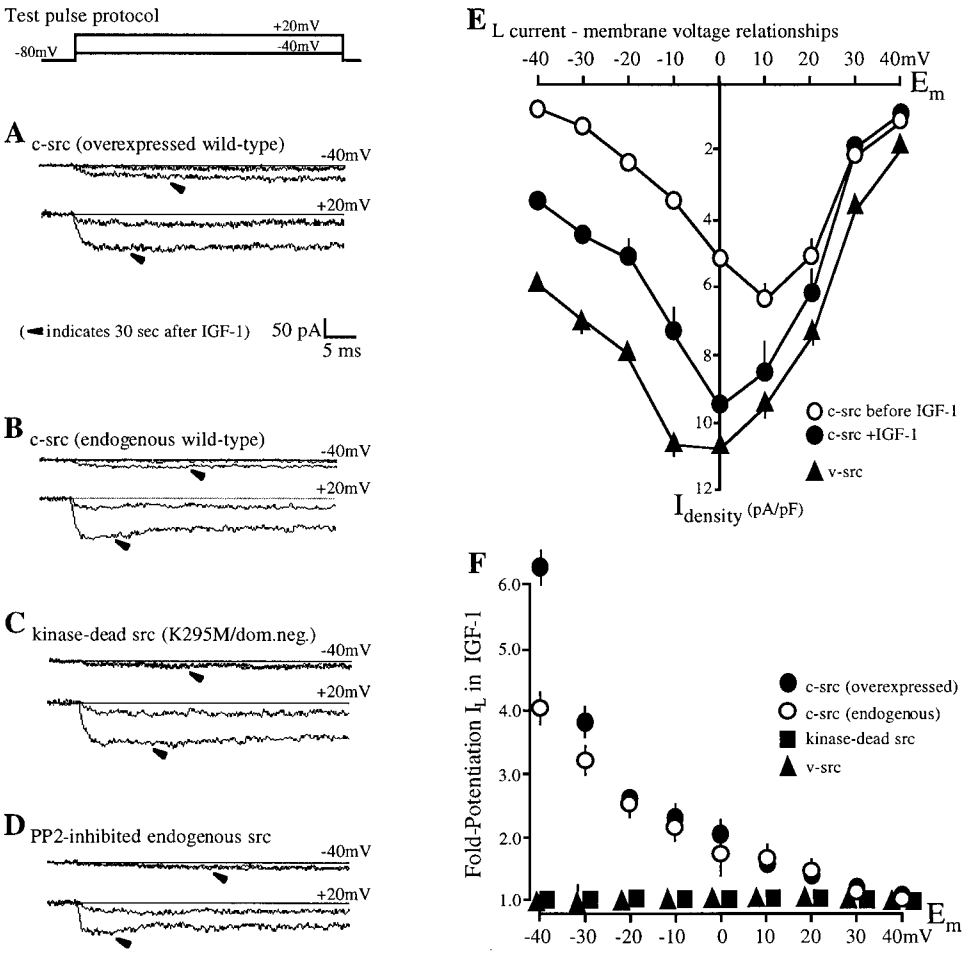

Figure 2. Rapid IGF-1 Potentiation of Cerebellar L Channels Requires a Src Family Tyrosine Kinase

(A) Overexpression of wild-type c-Src allows the normal, hyperpolarization-dependent IGF-1 potentiation of L channels and the normal, depolarization-dependent potentiation of N channels. Total n for neurons overexpressing c-Src, 18 of 18.

(A, C, and D) Each panel shows results from an individual neuron that was either transfected with a Src isoform (c-Src or kinase-dead Src, [A and C]) or acutely exposed to a Src family kinase inhibitor (D). Barium currents were evoked at two membrane potentials (−40 and +20 mV) before and 30 s after IGF-1 (20 ng/ml) addition (arrowheads). Test protocol is shown over (A).

(B) In untransfected neurons, IGF-1 potentiates L channel activity at hyperpolarized potentials. Total n, 9 of 9 neurons.

(C) Mutation of the ATP binding site renders Src inactive, and, as shown by the absence of potentiation only at hyperpolarized potentials, blocks IGF-1 L channel potentiation but not that of the N channels. Total n, 34 of 34 neurons.

(D) Brief pretreatment with the membrane-permeable Src family kinase inhibitor PP2 (10 μM) also uniquely blocks L channel potentiation. Total n, 7 of 7 neurons.

(E) In neurons overexpressing c-Src, IGF-1 strongly potentiates L channel currents at hyperpolarized membrane potentials, while overexpression of the constitutively active v-Src appears to mimic the IGF-1 effect; N currents were inhibited with ω-conotoxin-GVIA. L channel current–voltage relationships were determined for c-Src-transfected neurons before (open circles) and 30 s after (20 ng/ml, closed circles) IGF-1 addition and for v-Src-transfected neurons in the absence of IGF-1 (triangles). For each cell, all current levels were normalized to cell size, as estimated by membrane capacitance. Values are means ± SEM for n = 18 c-Src-expressing cells and n = 40 v-Src-expressing cells; where no bars are shown, the errors are smaller than the symbols denoting their means; at 0 mV, the values for c-Src neurons before and after IGF-1 are 5.2 ± 0.4 and 9.5 ± 1.2 pA/pF, and for v-Src-expressing neurons are 10.7 ± 0.7 pA/pF.

(F) Fold potentiation of L currents by IGF-1 in neurons expressing kinase-dead Src, v-Src, and endogenous and overexpressed wild-type c-Src: only cells expressing c-Src potentiate L currents at hyperpolarized potentials. Moreover, c-Src overexpression increases potentiation. At each test potential, the current measured after 20 ng/ml IGF-1 was divided by that measured before. Values are means ± SEM; where no bars are shown, errors are smaller than the symbols denoting the means; n for kinase-dead Src = 34 cells; n for c-Src = 18 cells; n for v-Src = 40 cells.

quences that might accompany longer-term overexpression studies. Untransfected neurons were pretreated for 20 min with 10 μM PP2 or its inactive analog, PP3, and IGF-1 potentiation was assessed as above. In no case was IGF-1 able to potentiate L currents in the presence of PP2 (Figure 2D; n = 7 of 7 cells), even though in all cases in which N currents were present, N channel potentiation occurred (Figure 2D; n = 5 of 5 cells). In addition, to ensure that any response would be detected, L currents were assessed for up to 10 min after IGF-1 addition, and, in some cases, the IGF-1 concentration was elevated to 100 ng/ml. Importantly, L channel potentiation took place normally in the presence of the inactive analog, PP3 (n = 5 of 5 cells).

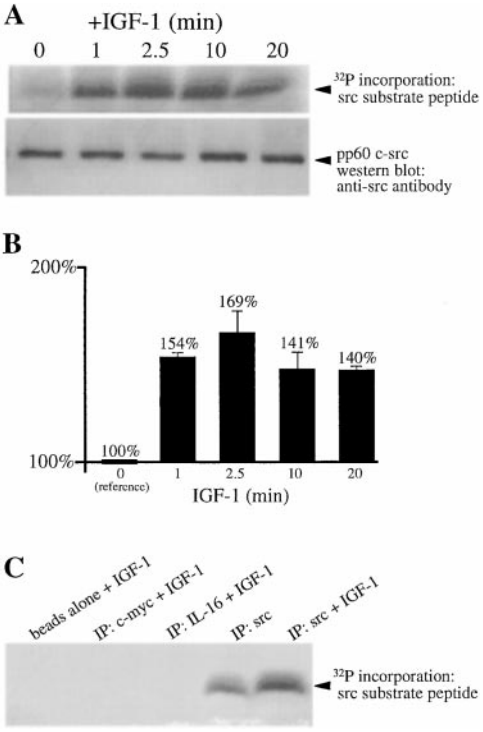

Figure 3. IGF-1 Rapidly Stimulates Src Catalytic Activity in Neuroblastoma Cells

(A) Time course of IGF-1 activation of Src in SH-SY5Y cells was assessed by immunocomplex kinase assay. Cells were stimulated with IGF-1 (50 ng/ml) as indicated and lysed, and endogenous Src kinase was immunoprecipitated from whole-cell extracts with a monoclonal anti-Src antibody (clone GD11). Protein G agarose–Src complexes were washed and reconstituted in Src kinase buffer, a Src substrate peptide ($p34^{cdc2}$, amino acids 6–20), and 10 μCi [γ^{32}P]ATP. Src activity was measured by ^{32}P incorporation into the Src substrate peptide and visualized by autoradiography.

(B) Cumulative data (means ± SEMs) from three independent experiments. The value obtained in the absence of IGF-1 stimulation (0 min) was defined as 100%.

(C) Immunocomplex kinase assay: control immunoprecipitations from SH-SY5Y cells. All cells (except in lane 4) were stimulated as in (A) for 2.5 min with IGF-1. Lane 1, protein G agarose beads alone. Lanes 2 and 3, nonrelevant monoclonal antibodies fail to precipitate Src from IGF-1-stimulated cells. Lanes 4 versus 5, as in (A), IGF-1 increases the activity of endogenous Src precipitated by the monoclonal anti-Src antibody.

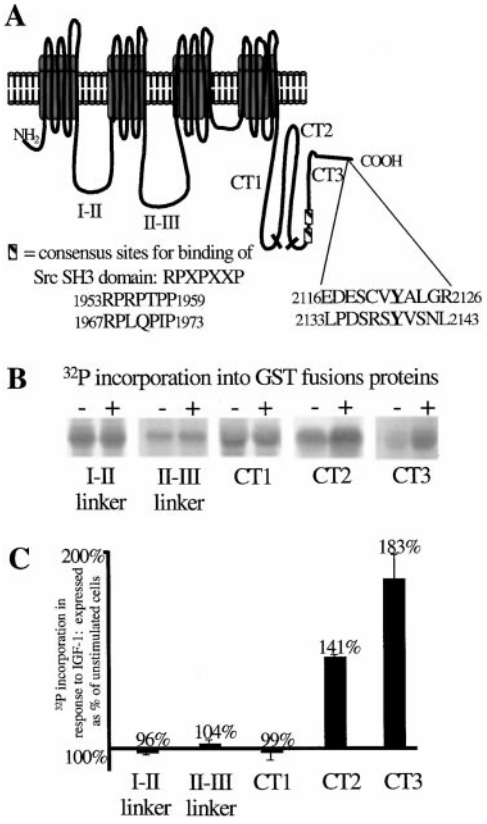

Figure 4. Specific Regions of α1C Increase ^{32}P Incorporation in Response to IGF-1 When Assayed In Vitro

(A) Diagram of GST fusion proteins encoding intracellular regions of the neuronal α1C subunit. The I–II linker encodes amino acids 405–525; the II–III linker, amino acids 754–903; CT1, amino acids 1480–1700; CT2, amino acids 1701–1931; and CT3, amino acids 1932–2143.

(B) ^{32}P-incorporation into the GST fusion proteins demonstrates that CT2 and CT3 are phosphorylated in response to IGF-1 (50 ng/ml, 3 min); 10 μg of each fusion protein was mixed with extracts of SH-SY5Y cells exposed to vehicle (−) or IGF-1 (+), bound to glutathione 4B sepharose beads, washed extensively, incubated in kinase buffer, and subjected to an in vitro kinase assay. Fusion proteins were resolved by SDS–PAGE and subjected to autoradiography.

(C) Levels of ^{32}P incorporation, as determined by phosphorimaging analysis. Shown are means ± SEMs from three independent experiments. Each value was calculated as the percent change relative to each unstimulated control.

We also found that at hyperpolarized potentials, basal L currents, recorded prior to IGF-1 addition, were substantially greater in neurons transfected with the constitutively active v-Src (Figure 2E). Moreover, subsequent superfusion of IGF-1 was incapable of inducing further increases (Figure 2F). Again, to ensure that no response was overlooked, IGF-1 concentrations were elevated and L currents recorded for extended periods. For N currents, though, v-Src failed to block IGF-1 potentiation ($n = 8$ of 8 cells), indicating that the Src effect is specific for L currents. Together, the results suggest that v-Src can induce full L channel potentiation, mimicking IGF-1 by bypassing the activation of upstream signaling components. Interestingly, the increase in basal L currents in v-Src neurons is greater than the IGF-1 potentiation observed in c-Src-transfected neurons (Figure 2E) or untransfected neurons 30 s after IGF-1 addition (Blair and Marshall, 1997), raising the possibility that the increased kinase activity associated with v-Src drives potentiation to (or toward) the maximum.

IGF-1 Rapidly Stimulates Src Catalytic Activity in SH-SY5Y Cells

Many growth factors activate Src family kinases in nonneuronal cells (reviewed by Thomas and Brugge, 1997). To determine if Src is rapidly stimulated by IGF-1 in our system, we performed a time course and measured the activity of immunoprecipitated Src using a substrate peptide demonstrated to be highly specific for Src family kinases ($p34^{cdc2}$, amino acids 6–20, and [γ^{32}P]ATP; Cheng et al., 1992). As shown in Figure 3, endogenous Src is rapidly activated by IGF-1 (50 ng/ml). An increase is

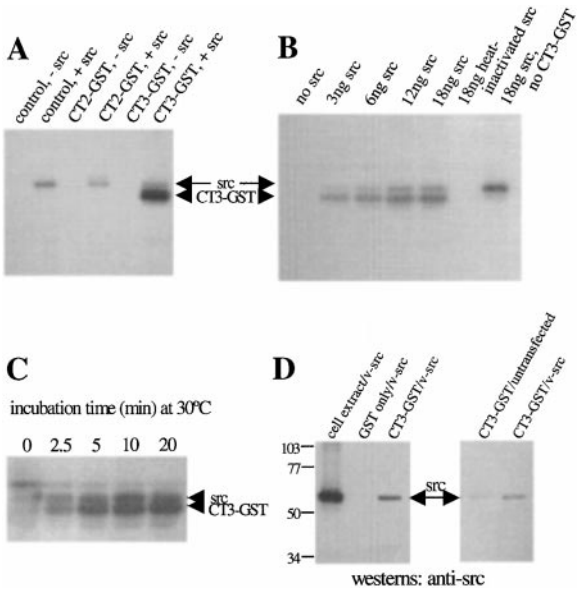

Figure 5. Purified Src Kinase Phosphorylates the CT3 Region of Neuronal α1C In Vitro

(A) Only the CT3-GST fusion protein (amino acids 1932–2143) is a substrate of Src enzyme in vitro. Autoradiogram of a kinase assay testing the ability of Src to phosphorylate GST fusion proteins encoding the CT2 and CT3 regions of the α1C C terminus. Fusion proteins (2 μg) dialyzed in Src kinase buffer were reconstituted with Src kinase buffer, pp60c-Src enzyme (UBI), and [γ^{32}P]ATP. No fusion protein is present in control lanes 1 and 2. Reactions were for 20 min at 30°C; proteins were separated on an 8% SDS–PAGE gel and subjected to autoradiography; n = 4 independent experiments.

(B) Dosage analysis of Src phosphorylation of CT3-GST. Autoradiogram of a kinase assay performed as in (A) with CT3-GST as the substrate shows that increasing the levels of Src kinase increases phosphorylation. Lane 6 shows the assay performed with 18 ng of heat-inactivated Src (5 min, 65°C). Lane 7 contains no fusion protein substrate; n = 3 independent experiments.

(C) Time course demonstrates increasing phosphorylation of CT3-GST with longer incubation times. Autoradiogram of an assay performed as above with CT3-GST as the substrate and increasing incubation times; n = 3 independent experiments.

(D) v-Src, as well as endogenous c-Src, binds to CT3-GST in vitro. Left panel, equimolar amounts of GST alone (lane 2) or CT3-GST (lane 3) were mixed with extracts of SH-SY5Y cells overexpressing exogenous v-Src. Lane 1, control cell extract (20 μg). Right panel, CT3-GST was mixed with extracts of untransfected cells (endogenous c-Src, lane 1) or of cells overexpressing exogenous v-Src (lane 2). As expected, more Src binding is detected when Src proteins are overexpressed. Fusion proteins were collected with glutathione 4B sepharose beads and washed extensively in high salt buffer to remove nonspecific binding; Western blots were probed with a monoclonal anti-Src antibody; n = 4 independent experiments.

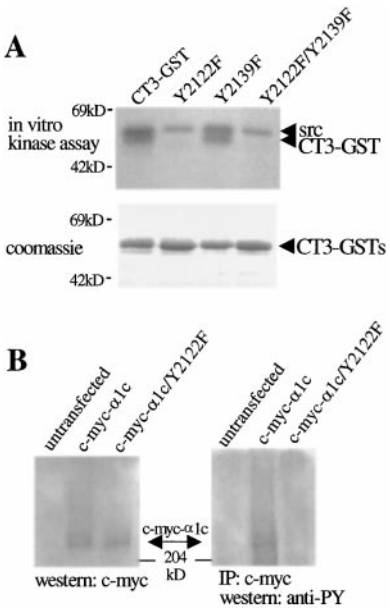

Figure 6. Src Kinase Phosphorylates Neuronal α1C on Tyrosine 2122: Analysis of Y2122F and Y2139F Mutants

(A) Purified Src kinase phosphorylates (Y2139F)CT3-GST to the same extent as wild-type CT3-GST. ^{32}P incorporation was assessed as in Figures 5B and 5C. The low level of incorporation into the double mutant Y2122F/Y2139F, which lacks all tyrosine residues, indicates the level of nonspecific, background labeling. ^{32}P incorporation into (Y2122F)CT3, as measured by phosphorimage analysis, was not different from background ($p < 0.2$, t test), while (Y2139F)CT3 did not differ from wild-type CT3 ($p < 0.4$). Bottom panel shows that equal amounts of all forms of the fusion proteins were used; n = 4 independent experiments.

(B) Full-length α1C/Y2122F is not tyrosine phosphorylated in response to IGF-1 in neuroblastoma cells. c-Myc-α1C and c-Myc-α1C/Y2122F were expressed in SH-SY5Y cells. Cells were stimulated with IGF-1 (3 min, 50 ng/ml) and α1C immunoprecipitated from soluble membranes with a c-Myc antibody. Untransfected cells were used as a control (lane 1, both panels). Left panel, control Western blot shows equal amounts of c-Myc-α1C and c-Myc-α1C/Y2122F. Right panel, subsequent Western blotting with anti-phosphotyrosine antibody demonstrates the inability of IGF-1 to phosphorylate the Y2122F mutant; n = 2 independent experiments.

detected in cells stimulated for as briefly as 1 min and is maintained for at least 20 min.

IGF-1 Increases ^{32}P Incorporation into the Neuronal α1C C Terminus

To assess which region(s) of α1C are phosphorylated in response to IGF-1, glutathione S-transferase (GST) fusion proteins were constructed to encode the major intracellular regions (Figure 4A). To allow any kinases that may be stimulated by IGF-1 to bind the various α1C regions, the fusion proteins were exposed to whole-cell extracts made from either unstimulated or IGF-1-stimulated SH-SY5Y cells. Fusion proteins were then pelleted with glutathione 4B sepharose beads and subjected to stringent washes. Beads and attached proteins were subsequently incubated under kinase assay conditions with [γ^{32}P]ATP to allow any attached kinases to phosphorylate the fusion protein. Change in ^{32}P incorporation into each fusion protein in response to IGF-1 was expressed as percent of control (incorporation into fusion proteins mixed with extracts of unstimulated cells). As shown in Figures 4B and 4C, the fusion proteins encoding regions CT2 and CT3 of the α1C C terminus increase significantly in ^{32}P incorporation in response to IGF-1: mean $\pm$ SEM = 141% $\pm$ 1%, and 183% $\pm$ 13%, respectively. There is no significant change in the I–II linker, II–III linker, or the CT1 fusion protein: 96% $\pm$ 1%, 104% $\pm$ 2%, 99% $\pm$ 4%, respectively. These results show that two distinct regions of the α1C C terminus are phosphorylated in response to IGF-1. In addition, these results indicate that the kinase(s) responsible for phosphorylating these regions must also associate with the channel.

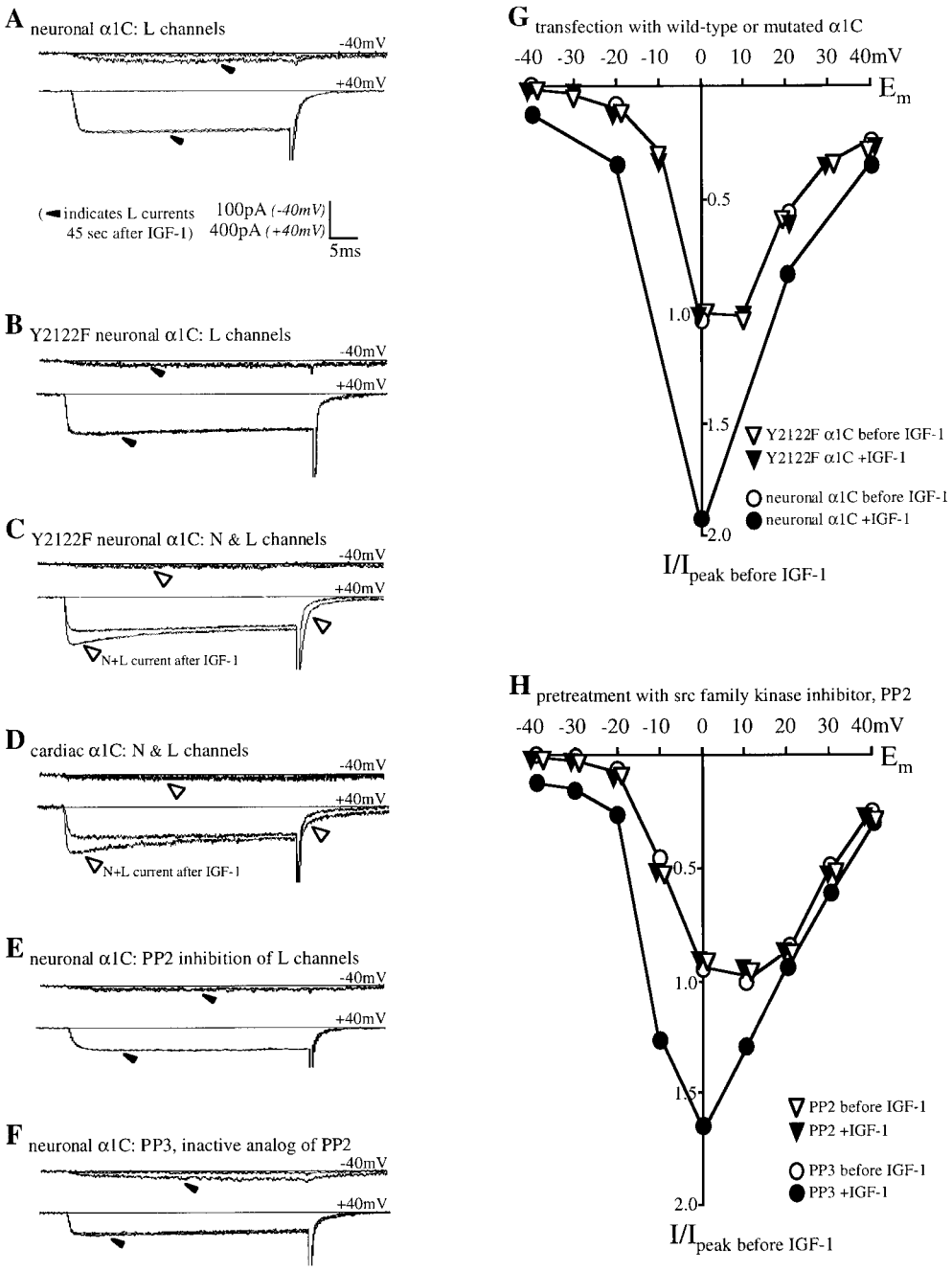

Figure 7. Phosphorylation of Y2122 Is Critical for IGF-1 Potentiation of Neuronal α1C L Channels

(A) Coexpression of wild-type neuronal α1C with L channel auxiliary subunits in SH-SY5Y cells leads to hyperpolarization-dependent L channel potentiation indistinguishable from that observed in neurons. Total n for wild-type neuronal α1C, 34 of 34 cells. Recordings were performed as in Figure 2. In (A) and (B), and (E) and (F), N currents were blocked with ω-conotoxin-GVIA, and the solid arrowheads indicate L currents recorded 45 s after IGF-1 (20 ng/ml) addition. In (C) and (D), N currents were present, and the open arrowheads indicate the combined N and L currents recorded 45 s after IGF-1 addition.

(B and C) Point mutation of Y2122 eliminates IGF-1 potentiation of L channels.

(B) With only L currents present, no potentiation occurs; total n = 0 of 15 cells.

(C) When the endogenous N currents are not blocked, expression of the Y2122F/neuronal α1C prevents the hyperpolarization-dependent L channel potentiation but allows N channel potentiation (total n = 8 of 8 cells), as shown by the increased transient component at depolarized potentials and an increase in a slower component of the tail current (see open arrowheads at +40 mV).

(D) IGF-1 cannot potentiate cardiac α1C L channels. Total n = 0 of 9 cells. Although the cardiac α1C subunit is highly homologous to its neuronal counterpart, it lacks a tyrosine at the corresponding position. Again, endogenous N channel activity was left intact (total n = 4 of 4 cells) to prove that cells had not lost the capability to respond; as shown by the open arrowheads at +40 mV, IGF-1 increases the evoked transient current and the slower component of the tail current.

Purified pp60c-Src Phosphorylates the CT3 Region of the α1C C Terminus, and Src Associates with a CT3-GST Fusion Protein

Since both the CT2 and CT3 regions increase in ^{32}P content in response to IGF-1, we tested the ability of Src kinase to phosphorylate these fusion proteins directly in an in vitro kinase assay. We find that Src directly phosphorylates the CT3 region of the channel but not CT2 (Figure 5A). Increasing the dosage of Src enzyme increased CT3 phosphorylation accordingly (Figure 5B, lanes 1–5). As a control, heat-inactivated Src was used (Figure 5B, lane 6); it showed no activity toward CT3 nor the Src autophosphorylation seen in the other lanes. When the CT3 fusion protein was omitted from the assay, only Src autophosphorylation is seen (Figure 5B, lane 7). Similarly, increasing incubation times also increased phosphorylation of CT3 (Figure 5C). We conclude that the CT3 region is a good substrate for Src kinase.

Since the ^{32}P incorporation experiments shown in Figure 4 suggest that the kinase(s) responsible for phosphorylation are associated with the channel, we tested whether an activated form of Src, v-Src, could bind CT3. Here, the CT3-GST fusion protein was mixed with extracts of v-Src-transfected SH-SY5Y cells; GST alone was used as a control. The proteins were collected, washed repeatedly under stringent conditions, and probed by Western blotting with a monoclonal anti-Src antibody (clone GD11). We find that both v-Src and endogenous c-Src bind to the CT3-GST fusion protein but not to GST alone (Figure 5D).

Src Kinase Phosphorylates the CT3 Region on Tyrosine 2122

There are two tyrosine residues within the CT3 sequence, Y2122 and Y2139 (Figure 4A). To determine which one the Src enzyme phosphorylates, both were mutated by site-directed mutagenesis. The most conservative substitution, phenylalanine, which differs structurally only in substituting a hydrogen for a hydroxyl, was chosen to minimize allosteric effects.

CT3-GST fusion proteins with the following mutations were purified and dialyzed into Src kinase buffer: Y2122F, Y2139F, and Y2122F/Y2139F. As shown in Figure 6A, we find that Src phosphorylates wild-type CT3-GST and (Y2139F)CT3-GST but does not phosphorylate (Y2122F)CT3-GST above background, indicating that Y2122 is the unique phosphorylation site. Furthermore, mutation of Y2122 in full-length Myc-α1C results in loss of IGF-1-induced tyrosine phosphorylation in intact SH-SY5Y cells (Figure 6B). Interestingly, upstream of Y2122 are several acidic amino acid residues (EDE...Y); based on sequences of known Src substrates (Pearson and Kemp, 1998), this motif may be favorable for Src phosphorylation.

Tyrosine 2122 of Neuronal α1C Is Critical for IGF-1 Potentiation

To determine whether phosphorylation of Y2122 regulates IGF-1/L channel potentiation, full-length neuronal α1C was coexpressed with the L channel auxiliary subunits α2δ and β1b in SH-SY5Y cells. Importantly, although these cells express significant N current, endogenous L currents are barely detectable, with peak levels of $<$10 pA (7 $\pm$ 1 pA, n = 15 untransfected cells). We also find that the transfected L channels express well, producing peak currents of 200 pA–2 nA, and that the voltage-dependent kinetics are normal, with relatively slow rise times ($\sim$2 ms) at hyperpolarized potentials dropping to $<$1 ms at depolarized potentials (Fox et al., 1987; Tomlinson et al., 1993; Randall and Tsien, 1995). IGF-1 potentiated wild-type neuronal α1C L channels in all cells (Figures 7A and 7G; n = 34 of 34 cells).

Importantly, IGF-1 failed to potentiate neuronal α1C L channels containing the Y2122F mutation (Figures 7B, 7C, and 7G; n = 0 of 15 cells), even though the cells were capable of N channel potentiation (Figure 7C; n = 8 of 8 cells), and the currents were otherwise indistinguishable from those produced by wild-type α1C. The ability of IGF-1 to potentiate cardiac α1C L channels was also tested. Although $\sim$95% identical to the neuronal isotype, cardiac α1C lacks Y2122 and was not potentiated by IGF-1 (Figure 7D; n = 0 of 9 cells); in the same cells, N currents were potentiated (Figure 7D; n = 4 of 4 cells). We additionally assessed if inhibition of Src family kinases could prevent modulation of wild-type neuronal α1C. As with the cerebellar neurons, acute pretreatment with 10 μM PP2 prevented IGF-1 potentiation of wild-type neuronal α1C L channels in all cases (Figures 7E and 7H; n = 10 of 10 cells), while pretreatment with the inactive analog, PP3 (10 μM), had no effect (Figures 7F and 7H; n = 8 of 8 cells).

To determine the biophysical mechanism of modulation, the rates of L channel activation and deactivation were estimated. Wild-type neuronal α1C, Y2122F/neuronal α1C, and cardiac α1C L currents were analyzed. We find that the chief kinetic effect of IGF-1 on wild-type neuronal α1C currents is an increased rate of activation at hyperpolarized potentials. At −40 and −30 mV, L currents rose three to four times faster within 45 s of IGF-1 addition (fold change in τ_{rise} [mean $\pm$ SEM] = 3.2 $\pm$ 0.2 at −30 mV), but little or no change occurred at potentials $\geq$+20 mV (n = 5 cells). No differences in deactivation (i.e., channel closure), as indicated by tail

(E and F) Acute pretreatment with the Src family kinase inhibitor PP2 similarly blocks IGF-1/L channel potentiation (E), while its inactive analog, PP3, does not (F). SH-SY5Y cells expressing normal neuronal α1C subunit were pretreated for 20 min with either PP2 or PP3 (each at 10 μM), and L channel currents before and after IGF-1 were assessed as in (A). Total n, 10 of 10 PP2-treated cells, 8 of 8 PP3-treated cells.

(G) L channel current–voltage relationships for the wild-type and Y2122F neuronal α1C-expressing cells shown in (A) and (B): only the cell expressing wild-type α1C responds; in the Y2122F α1C-expressing cell, L currents before and after IGF-1 overlap with each other and with those of the cell expressing wild-type α1C before IGF-1. Note that although L currents are small at hyperpolarized potentials, the fold increase in the cell expressing wild-type α1C is much greater at −40 mV than at potentials $\geq$0 mV. Additionally, because α1C-transfected cells express different L current levels, L currents measured at each potential before and after IGF-1 were divided by the peak current measured before IGF-1; peak L current values were 1.7 nA for the wild-type α1C-expressing cell and 1.02 nA for the Y2122F α1C cell. N currents were blocked with ω-conotoxin-GVIA.

(H) Effects of the Src family kinase inhibitor PP2 and its inactive analog, PP3: L current–voltage relationships for the wild-type neuronal α1C-expressing cells shown in (E) and (F) reveal that only the active inhibitor blocks IGF-1 potentiation. L currents were normalized as in (G).

currents, were detected (Figures 7A and 7F). It should be noted that in Figures 7C and 7D, where no Y2122F/neuronal α1C and cardiac α1C potentiation occurred, the tail currents increased due to IGF-1 potentiation of endogenous N channels. Our results suggest that a primary effect of IGF-1-modulation is an increased probability of L channel opening at hyperpolarized membrane potentials.

We also find that, prior to IGF-1 addition, the rise times of both Y2122F/neuronal α1C ($n = 4$ cells) and cardiac α1C L channels ($n = 3$ cells) are identical to those of the wild-type neuronal α1C L currents. Significantly, however, superfusion of IGF-1 failed to alter their rise times at any potential: by comparing times before and after IGF-1 ($\tau_{rise\text{-}before}$ divided by $\tau_{rise\text{-}after}$ to obtain the fold change at each test potential), changes were negligible, varying from 0.94 ± 0.04 to 1.04 ± 0.04. Intriguingly, the increased rates of wild-type neuronal L channel activation induced by IGF-1 appear to shift the current–voltage relationship. For the endogenously expressed cerebellar L channels and the heterologously expressed wild-type neuronal α1C channels, peak currents prior to IGF-1 treatment are typically obtained at $+10$ mV (see Figures 2E, 7G, and 7H). Following IGF-1 potentiation, the peak shifts to $\sim$0 mV (Figures 2E and 7H), an effect that does not occur when potentiation is blocked by either Src inhibition (Figure 7H) or expression of the Y2122F/neuronal α1C mutant (Figure 7G).

Discussion

Our results demonstrate that Src or a related Src family kinase mediates the rapid IGF-1 potentiation of neuronal L channels via phosphorylation of a specific C-terminal tyrosine residue, Y2122, on the α1C subunit. This site is not present in the cardiac α1C, which IGF-1 fails to potentiate, suggesting that the cardiac muscle channel could be regulated differently.

The essential role of a Src family tyrosine kinase in IGF-1 modulation was demonstrated biochemically and electrophysiologically. Purified Src kinase or extracts of IGF-1-stimulated cells were employed, as well as in vitro kinase assays using fusion proteins encoding intracellular regions of α1C. We show that IGF-1 stimulation induces phosphorylation of two regions of the α1C C terminus; that Src can associate with the most C-terminal region of α1C, CT3; that purified Src phosphorylates CT3 in vitro; and that IGF-1 stimulates Src activity in intact cells. Interestingly, two stretches of amino acids in CT3 conform to the canonical Src SH3 domain binding site RPLPXXP (Sparks et al., 1996).

How phosphorylation of an individual subunit alters ion channel physiology is unclear. It is possible that phosphorylation of the C terminus may directly cause a conformational change of the α1C subunit, changing the kinetics of channel behavior. On the other hand, it may alter interactions between α1C and adaptor protein(s), indirectly modifying channel physiology. Other calcium channels have been shown to associate with a variety of adaptor and anchoring proteins. Moreover, A kinase–anchoring proteins, AKAPs, regulate cardiac L channel phosphorylation and activity (Gao et al., 1997) and associate with muscle L channels (Gray et al., 1998).

Interestingly, we find that overexpression of v-Src uniquely affects basal L currents, inducing at hyperpolarized potentials a substantial increase that is greater than the IGF-1 potentiation observed in c-Src-transfected neurons immediately after IGF-1 addition. One possible explanation is that v-Src-driven increases in calcium influx may stimulate de novo L channel synthesis (Murphy et al., 1991; Wang et al., 1999). Another, which does not exclude the first, is that the increased kinase activity associated with v-Src drives potentiation toward the maximum. The direct tyrosyl phosphorylation of α1C and association of Src with the α1C C terminus favor this hypothesis, as do our results demonstrating that when overexpressing rate-limiting intermediates, the IGF-1 potentiation increases (Blair and Marshall, 1997). However, v-Src can also phosphorylate and activate IGF-1 receptors (Kozma and Weber, 1990; Peterson et al., 1996; Arbet-Engels et al., 1999). Therefore, endogenous c-Src, once activated, might act via both direct and indirect mechanisms, creating a positive feedback cycle that would be predicted to drive L channel potentiation to higher levels. Recurrent Src stimulation of the full pathway could be particularly important if increased L channel activity required, in addition to Src-dependent α1C phosphorylation, one or more parallel events. Our results showing that CT2 can be phosphorylated by a non-Src kinase support the idea that multiple phosphorylation events may be essential.

IGF-1- and Src-dependent modulation of L channels may regulate a variety of cellular processes. Potentiating L channels is known to induce transcription (reviewed by Finkbeiner and Greenberg, 1996), and L channel–mediated influx during embryonic calcium action potentials is essential for neurite outgrowth, synapse formation, and the developmental shift to mature, sodium-dependent action potentials (reviewed by Spitzer and Ribera, 1998). Interestingly, developing granule neurons make calcium action potentials from generation till synapse formation (D'Angelo et al., 1997), and L channel antagonists decrease granule neuron survival (Gallo et al., 1987; Galli et al., 1995; Blair et al., 1999a; Leski et al., 1999). However, IGF-1 is strongly neuroprotective (reviewed by Apfel, 1999), partially through a pathway requiring IGF-1/L channel potentiation (Barale et al., 1998; Blair et al., 1999a). We observe an IGF-1- and Src-induced increase in L channel activation uniquely at hyperpolarized potentials. This voltage dependence may be crucial for the cellular outcome. Recent work shows that relatively little influx may influence transcription (Jacobs and Meyer, 1997), suggesting that even minor changes in L channel activation could be critical, leading to increased probability of firing calcium action potentials and influencing differentiation. Subthreshold excitatory activity might trigger calcium influx, regulating survival and, potentially, numerous other cellular processes.

Experimental Procedures

Cell Culture

Cerebellar granule neurons and neuroblastoma cells were prepared as previously described (Blair et al., 1999b). To eliminate preexposure to growth factors, we switched cells to zero-serum Dulbecco's modified Eagle's medium (DMEM) for 4–16 hr prior to testing. Human recombinant IGF-1 was from Calbiochem.

Expression Vectors and Transfection

c-Src and c-Src(K295M) cDNAs were in pM5HHB5 expression vector under LTR promoter control. v-Src cDNA was in PRK5 vector under cytomegalovirus (CMV) promoter control (Moss et al., 1995).

cDNAs encoding rat brain α1C, α2δ, and β1b subunits were in CMV-based vertebrate expression vectors. α1C cDNA was amplified by PCR and subcloned into pCS2MT (SV40 promoter), placing the c-Myc tag in-frame at the C terminus. All clones were confirmed by DNA sequencing.

Granule neurons were transfected by calcium-phosphate (Blair and Marshall, 1997; Blair et al., 1999b) with 0.2 μg cDNAs encoding c-Src, c-Src (K295M), or v-Src and 2 μg SuperGlo- (sg25-) GFP cDNA (Quantum Biotechnologies). SH-SY5Y cells were transfected by lipid-mediated transfection (Blair et al., 1999b) using 4–6 μg cDNAs of interest plus 1 μg large T antigen (pRSV-LT) cDNA and LipofectAMINE-PLUS (GIBCO–BRL). For biochemistry, cells were harvested 48 hr posttransfection, except v-Src-transfected cells, which were harvested within 24 hr. The cDNAs encoding α1C, α2δ, and β1b (or β2a) calcium channel subunits were transfected in a 2:1:1 ratio. For recordings, 1 μg GFP cDNA was added.

Immunoprecipitation and Immunoblot

Following a 3 min IGF-1 stimulation, cells were homogenized on ice in 140 mM NaCl, 10 mM NaHEPES (pH 7.4), 5 mM EGTA, and 5 mM EDTA. Membranes were pelleted (39,000 rpm, 2 hr) and solubilized in phosphate-buffered saline (PBS), 1% NP-40, 0.5% Na-deoxycholate, 0.5% SDS, 1.2% Triton X-100, 5 mM EGTA, and 5 mM EDTA (4°C, 2 hr). Insoluble membranes were pelleted (50,000 rpm, 1 hr). Protein concentration of soluble membranes was measured with the Bio-Rad DC kit. All buffers contained protease and phosphatase inhibitors: 50 mM NaF, 0.5 mM sodium orthovanadate, 100 nM okadaic acid, 100 nM calyculin A, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml benzamidine, and 8 μg/ml calpain inhibitor I and II.

Endogenous α1C subunits were immunoprecipitated overnight at 4°C from 750 μg solubilized granule cell membranes with the CARDI antibody (Chien et al., 1995). Heterologously expressed Myc-α1C subunits were immunoprecipitated overnight at 4°C from 300 μg solubilized SH-SY5Y membranes with a monoclonal c-Myc antibody (clone 9E10, Oncogene Research Products). Protein G PLUS agarose beads (Santa Cruz Biotechnology) were added, and samples were rotated (4°C, 1 hr). Complexes were pelleted and washed twice in 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.5% NP-40, and 1% Triton X-100 and twice in 20 mM Tris (pH 7.4), 1 mM EGTA, and 1 mM EDTA. Beads were resuspended in 2× Laemmli sample buffer and sonicated briefly on ice. Proteins were separated on 5% SDS–PAGE gels with a 3.5% stacking gel and transferred for Western blotting.

Membranes were blocked for 1 hr in Tris-buffered saline (TBS)/5% milk, except for phosphotyrosine blots, which were blocked in TBS/1% milk/1% bovine serum albumin/0.01% Tween-20, then exposed to primary antibody (c-Myc 9E10 mAb, Oncogene Research Products; anti-phosphotyrosine 4G10 mAb, UBI). Membranes were washed with TBS/0.1% Tween-20 and exposed to secondary antibody (anti-mouse IgG-HRP, Transduction Laboratories). Bands were visualized by enhanced chemiluminescence (Amersham Pharmacia).

Electrophysiology

Either standard whole-cell patch recordings or the permeabilized patch variation of the standard whole-cell technique (Hamill et al., 1981; Rae et al., 1991) was employed with indistinguishable results. Calcium currents were recorded as previously described (Blair and Marshall, 1997; Blair et al., 1999a). For all cells, the pulse protocol was run a minimum of two to four times prior to IGF-1 addition to ensure that currents were stable. IGF-1, diluted into extracellular recording saline, was superfused over the cells. For granule neurons, currents were recorded for 10–500 s after IGF-1 superfusion. With α1C channels heterologously expressed in SH-SY5Y cells, peak currents were often ~2 nA, and rundown was a concern. Therefore, the pulse series was run less frequently (every 45 s), pulse duration was minimized (30–50 ms), and, in some cases, the number of pulses in a series was reduced; all cells showing >10% rundown prior to IGF-1 addition were eliminated. Curve fitting was done using HEKA software (Instrutech) allowed to run to 1,000 iterations; the rising phase was fit with a single exponential. At the end of each test pulse, the membrane potential was returned to the holding potential (−80 mV), revealing tail currents, which were fit with the sum of two exponentials. Because L channels show slow and little inactivation during a test pulse and because we employed short test pulses, it was not possible to determine if time-dependent reductions in currents took place during a pulse. Fits were confirmed by eye; currents that were not well fit or were too noisy to obtain a unique fit were eliminated.

L currents were isolated pharmacologically and by ion substitution. Na^+ current was blocked with tetrodotoxin (TTX); K^+ currents with tetraethylammonium chloride (TEA) and 4-aminopyridine (4-AP), and by substitution of intracellular (pipette) K^+ with cesium; N currents with ω-conotoxin-GVIA (500 nM, Sigma); and P and Q calcium channels with ω-agatoxin-IVA. Extracellular saline was 20 mM $BaCl_2$ (as the charge carrier), 100 mM NaCl, 20 mM TEA, 5 mM 4-AP, 1 μM TTX, 300 nM ω-agatoxin-IVA, and 10 mM NaHEPES (pH 7.4). ω-conotoxin-GVIA (500 nM) was sometimes included to inhibit N channel activity. Patch electrodes (Corning 8161 glass) were filled with (in mM): CsCl, 150; BAPTA, 5; and NaHEPES, 10 (pH 7.4), as well as, in some cases, amphotericin B (0.25 mg/ml). Src family kinases were blocked using the membrane-permeable inhibitor PP2 or its inactive analog, PP3 (Calbiochem); stock solutions (20 mM in DMSO) were sonicated into extracellular saline or media (final concentration, 10 μM). When Src isoforms were overexpressed, neurons were tested as soon as GFP fluorescence was detectable (16–20 hr posttransfection).

Immunocomplex Kinase Assay

IGF-1-stimulated SH-SY5Y cells were lysed in PBS, 0.1% SDS, 0.5% Na-deoxycholate, and 1% NP-40 plus protease inhibitors, as above, and whole-cell extracts were prepared by solubilizing at 4°C for 1 hr; insoluble material was pelleted (10,000 rpm, 10 min). Endogenous Src was immunoprecipitated from 500 μg whole-cell extract with 4 μg anti-Src monoclonal antibody (clone GD11, UBI); control (nonrelevant) antibodies were anti-IL-16 (4 μg, Pharmingen) and anti-c-Myc (4 μg, clone 9E10, Oncogene Research Products). Protein G PLUS agarose beads were added, pelleted, and then washed three times, as above, and once in Src kinase buffer (50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA, and 5 mM β-mercaptoethanol). The complex was resuspended in 25 μl Src kinase buffer, 10 μg Src substrate peptide ($p34^{cdc2}$, amino acids 6–20, resuspended in Src kinase buffer, UBI), and 10 μCi [γ^{32}P]ATP (~3,000 Ci/mmol). Reactions were for 25 min at 30°C and were stopped by adding 2× sample buffer. Samples were boiled and peptide substrate resolved on 20% SDS–PAGE gels. ^{32}P incorporation was visualized by autoradiography.

Construction and Purification of GST Fusion Proteins

Primers were designed to amplify the following intracellular regions of α1C: I–II linker, II–III linker, CT1, CT2, and CT3. GST fusion proteins were constructed by subcloning PCR products into the SalI–NotI restriction sites of pGEX-4T-1 (Pharmacia Biotech). All constructs were confirmed by DNA sequencing.

pGEX constructs were transformed into BL21 bacteria (Novagen). Cultures (500 ml) were grown in LB/ampicillin induced with 1 mM IPTG (4 hr, 37°C) and pelleted. Pellets were resuspended in 30 ml PBS/1% Triton X-100 and lysed on ice by French press (1260 psi). Insoluble material was pelleted (12,000 rpm, 30 min), and soluble fusion proteins were captured using glutathione 4B sepharose beads (Amersham Pharmacia). Beads were washed four times in ice-cold PBS/1% Triton X-100, and fusion proteins were eluted with 50 mM Tris and 5 mM reduced glutathione (pH 8.0). Depending on the application, fusion proteins were dialyzed into PBS or kinase buffer.

^{32}P Incorporation Assay

Sister cultures of SH-SY5Y cells were stimulated with IGF-1 (50 ng/ml, 3 min) or vehicle (DMEM alone). Cells were extracted as above. Insoluble material was pelleted (10,000 rpm, 10 min). Extracts (2 mg) were exposed to 10 μg purified α1C fusion proteins (2 hr, 4°C). Glutathione 4B sepharose beads were added, rotated for 1 hr, pelleted, washed twice at 4°C with 500 mM NaCl, 10 mM NaHEPES (pH 7.4), 0.4% NP-40, 5 mM EGTA, and 5 mM EDTA and twice with 150 mM NaCl, 10 mM NaHEPES (pH 7.4), 0.4% NP-40, 5 mM EGTA,

and 5 mM EDTA plus protease and phosphatase inhibitors, then resuspended in 30 μl kinase buffer (20 mM Tris [pH 7.4], 20 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM EGTA, 5 mM β-mercaptoethanol, and 1 μM ATP) and incubated (25 min, 30°C) with 10 μCi [γ^{32}P]ATP (Amersham Pharmacia). Reactions were stopped by adding 2× sample buffer, samples were boiled, and proteins were separated on a 10% SDS–PAGE gel. Bands were visualized by autoradiography, and ^{32}P incorporation was quantitated by phosphorimage analysis (Fujifilm Bio Imaging Analyzer).

In Vitro Kinase Assay

Purified recombinant $p60^{c\text{-}Src}$ (15 U or as indicated in the figures; 15 U transferred 1.42 pmol phosphate/min/U to 150 μM Src substrate peptide; UBI) was reconstituted in Src kinase buffer with 2 μg GST fusion protein and 10 μCi [γ^{32}P]ATP. Reactions were for 20 min at 30°C and were terminated by adding 2× sample buffer. Proteins were separated on 10% SDS–PAGE gels and visualized by autoradiography.

Binding Assay

PRK5/v-Src was expressed in SH-SY5Y cells. Cells were extracted as above; 750 μg of cell extracts was mixed with equimolar amounts of GST or CT3-GST fusion protein (2 hr, 4°C). Glutathione 4B sepharose beads were added and processed as in ^{32}P incorporation assay protocol; proteins were separated on 8% SDS–PAGE gels, transferred, and probed by Western blotting for Src.

Site-Directed Mutagenesis

To create the CT3-GST tyrosine mutants Y2122F, Y2139F, and Y2122F/Y2139F, the CT3 region of α1C was amplified and subcloned into pGEX4T.1 as described above, substituting 3′ primers containing the base pair changes corresponding to the desired mutation. To create the full-length pCS2MT/α1C-Y2122F mutant, a 1.1 kb piece containing the desired tyrosine residue was cut from pCS2MT with XbaI and subcloned into pBluescript KS(−). Point mutation was made by QuikChange (Stratagene). Purified PCR product was subcloned into pCS2MT. All clones were confirmed by DNA sequencing.

Acknowledgments

We thank Dr. T. Snutch for the rat brain α1C, α2δ, and β1b clones; Dr. M. Hosey for CARDI antibody; Dr. E. Recio-Pinto for SH-SY5Y cells; Dr. T. Tanabe for the cardiac α1C clone; Dr. K. Campbell for β2a; Dr. T. Franke for pM5HHB5/c-Src and c-Src(K295M); Dr. N. Saccomano (Pfizer) for ω-agatoxin-IVA; Dr. I. Levitan and Dr. R. Huganir for PRK5/v-Src; and Drs. A. Ribera and D. Turner for the pCS2MT vector. This work was supported by National Institutes of Health grant RO1 NS37676-01A1 to L. A. C. B. and American Heart Association grant 9940131N to J. M.

Received February 18, 2000; revised May 2, 2000.

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